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RAPIDLY EVOLVING REPETITIVE DNA IN A KARYOTYPICALLY MEGAEVOLVED GENOME: FACTORS THAT AFFECT CHROMOSOMAL EVOLUTION

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Among some congeneric species of mammals, one taxon exhibits chromosomal stasis, whereas the other species possess a radically reorganized karyotype (Baker and Bickham, 1980). This phenomenon of radically reorganizing the G-banded pattern of the karyotype is termed karyotypic megaevolution and it involves an extensive amount of chromosomal evolution through the incorporation of both the numbers and kinds and types of rearrangements. The most classical example of karyotypic megaevolution is found in two species of *Muntiacus* where diploid numbers are 2n= 6 for *M. muntjak* and 2n = 46 for *M. reevesi* (Fredga, 1977). A significant aspect of this phenomenon is that the euchromatic regions of the genome can be changed substantially while having little or no affect at the phenotype (morphologic) level, at least not a sufficient amount of change to justify generic distinction. Karyotypic megaevolution probably represents the extreme condition for rate of karyotypic change involving complex chromosomal rearrangements (i.e. tandem fusions, peri and paracentric inversions). Relative to understanding the factors that affect chromosomal evolution, karyotypic megaevolution provides examples for studies involving two closely related species where one maintains chromosomal stasis while the other undergoes numerous chromosomal rearrangements.

Many different factors have been proposed to drive chromosomal evolution. These include demographic models (Wright, 1941; Wilson et al., 1975; Bush et al., 1977; Lande, 1979) and genetic and molecular factors (Pathak et al., 1973; Hsu et al., 1975; Hatch et al., 1976; Finnegan et al., 1982; Shaw et al., 1983; Wurster-Hill et al., 1988; Graphodatsky, 1989; Baker and Wichman, 1990; Meyne et al., 1990; Redi et al., 1990; Wichman et al., 1991). Of these hypotheses, only the tandem-repeat model by Wichman et al. (1991) establishes a set of testable predictions. Specifically, Wichman et al. (1991) proposed that lineages undergoing rapid karyotypic change would have multiple families of tandem repeats; whereas lineages characterized by karyotypic stasis would have fewer families and a lower abundance of tandem repeats. In addition, taxa possessing multiple repeats would be expected to have these repeats actively changing chromosomal fields, as suggested by Lima-De-Faria (1980), whereas in taxa expressing karyotypic stasis, these repeats would be restricted to a single chromosomal field. From a population genetics standpoint, litter-

size, effective population size, and generation time (i.e. demographic and populational characteristics) do not vary so radically in congeneric taxa that they could explain the extreme differences in rates and types of chromosomal rearrangements observed in karyotypic megaevolution.

The tandem-repeat model (Wichman et al., 1991) was based on studies of genome organization in equids. Equids have been proposed to be the most rapidly evolving karyotypic group of mammals (Wilson et al., 1975). Within the genome of six species of equids, it was found that families of tandem repeats were diverse and that the chromosomal fields (Lima-De-Faria, 1980) occupied by these repeats varied substantially among closely related species.

Using bats as a model, Bradley and Wichman (1994) tested the hypothesis that chromosomal evolution was associated with the occurrence of tandemly repeated DNA sequences. The phylogenetic screening method (Wichman et al. 1985, 1990) was used to evaluate the activity of tandemly repeated sequences in a conservatively evolving genome represented by the bat species *Macrotus waterhousii* compared to the activity of tandem repeats in the more rapidly evolving genome of the outgroup taxon (*Artibeus jamaicensis*). Their findings indicated that the number of families of repeated sequences were lower in *M. waterhousii* than in *A. jamaicensis*; thus providing initial support for the Wichman et al. (1991) predictions concerning karyotypic evolution. However, the study by Bradley and Wichman (1994) represented only one end of the comparative spectrum. What was lacking was an evaluation of the families and abundance of tandemly evolving repeats from a taxon with a radically reorganized karyotype (megaevolved) compared to a more conservatively evolving karyotype (chromosomal stasis).

In this study, tandemly repeated DNA sequences were examined from the bat species *Rhinophylla pumilio*. This species was selected for two reasons. First, *R. pumilio* possesses a radically evolved karyotype having accumulated > 20 rearrangements compared to its sister taxon *R. fischerae*. This extensive reorganization makes it impossible to compare to the G-banded karyotype of *R. pumilio* to the proposed primitive karyotype (*M. waterhousii*) for the family Phyllostomidae (Baker, 1979; Baker et al., 1989). On the other hand, *R. fischerae* has a karyotype that differs from the primitive condition by seven rearrangements: four fusions, one inversion, and two terminal translocations (Baker et al. 1987). This characteristic suggests that *R. pumilio* possesses one of, if not the most evolved karyotype in the family Phyllostomidae. Second, *R. pumilio* belongs to the same family (Phyllostomidae) as *A. jamaicensis* and *M. waterhousii* allowing for a comparison of tandemly repeated sequences in a known phylogenetic framework. Specifically, our goal was to compare the number of rapidly evolving tandemly repeated DNA sequences found in a species undergoing karyotypic megaevolution (*R. pumilio*) to that found in a taxon demonstrating chromosomal stasis (*M. waterhousii*). If the number of tandemly repeated sequences in *R. pumilio* statistically exceeds that found in *M. waterhousii*, then the hypothesis of Wichman et al. (1991) remains viable.

METHODS AND MATERIALS

High molecular weight DNA was isolated from approximately 0.5 g of liver tissue of *A. jamaicensis* (TK 32042, male, Cuba: Guantanoma Province; Guantanoma Bay Naval Base) and *R. pumilio* (TK 17565, female, Surinam: Marowijne; 3 km SW Albina). Methods for DNA isolation, cloning, digestion, transfer, and hybridization followed that of Bradley and Wichman (1994). Specifically, a genomic library was constructed from *R. pumilio* by generating partial digests of genomic DNA using the restriction enzyme *Sau*3AI. Digests were electrophoresed on low melting point agarose gels. DNA fragments in the 4-6 kb range were extracted, ligated into the *Bam*HI site of pUC 18 vector, and transformed by electroporation into the JM103 strain of *E. coli*.

Operationally, clones were not amplified during the transformation process; thus each clone represented a unique DNA fragment and was treated as such by assigning each an identification number. DNA from

each clone was triple digested with *Eco*RI, *Hin*dIII, and *Bam*HI, electrophoresed on 0.8% agarose gels, and DNA transferred to two positively charged nylon membranes (Boehringer Mannheim) by placing filters above and below the gel following a modified technique of Southern (1975).

This generated two identical membranes each with bound DNA from *R. pumilio* clones that were used for the phylogenetic screening procedure (Wichman et al., 1985). Each filter was hybridized to labeled genomic probes, one from *R. pumilio* (ingroup) and the other from *A. jamaicensis* (outgroup). Probes were constructed from genomic DNA labeled with [^{32}P]dCTP using random-primed labeling techniques. Hybridization conditions were standardized with those of Bradley and Wichman (1994) to provide a comparison among bat species, and to compare rates obtained from rodents (Wichman et al., 1990) and primates (Lloyd et al., 1987).

A second verification was performed, hybridizing the same membranes using the ECL Direct kit (Amersham). Membranes were washed previous to the hybridization in 1x SSC, 1%SDS at 65°C for one hour and rinsed two times in 2xSSC for five minutes at room temperature. Genomic DNA of *A. jamaicensis* and *R. pumilio* was sonicated and 1.5 µg were labeled. DNA was hybridized at 42°C overnight in 150 ml hybridization solution (ECL kit). Post-hybridization washes included two washes in 2xSSC for five minutes at room temperature, two washes in ECL post hybridization solution (6M urea, 04% SDS, 0.5xSSC) at 42°C for 10 minutes, and two washes in 2xSSC for five minutes. Membranes were blotted and submerged in reagents I and II for one minute and exposed on film.

Autoradiograms of the two sets of filters (from both experiments) were overlaid and compared for absence or presence of hybridization of *R. pumilio* or *A. jamaicensis* genomic DNA with the clone fragments. In addition, the difference in intensity was compared by scoring bands as faint, medium, or strong. Under these conditions, only repetitive sequences show detectable hybridization because single copy sequences are under-represented in a total genomic probe. The presence or absence of a band indicates the gain or loss of a repetitive element or portion of the element, whereas the difference in intensity indicates the number of copies of that element.

Clones identified as different between the ingroup and outgroup were verified by repeating the phylogenetic screening procedure and were designated as hypervariable. Hypervariable clones were sorted into families by Southern blot cross-hybridization (Southern, 1975). If clones possessed multiple bands (fragments), each band was numbered sequentially beginning with the largest fragment. Cross-hybridization experiments involved labeling the hypervariable band(s) of each clone and using it to probe the other hypervariable clones. The bands of interest were excised from 0.8% agarose gels, extracted and purified with the Prep-A-Gene kit (Bio-Rad), radiolabeled, hybridized, and scored as described above. These experiments were repeated until all clones were assigned to at least one family.

RESULTS

Phylogenetic screening methods (Wichman et al., 1985) were used to screen 747 clones from a genomic library constructed from *R. pumilio* DNA. Of these, 103 did not show any detectable hybridization to *A. jamaicensis* or self genomic DNA, indicating that they probably represented single or low copy sequences. The average insert size per clone was approximately 4.9 kb. If bats possess a genome size that is approximately 60-80% of the typical mammalian genome (Manfredi-Romanini, 1985; Burton et al., 1989), then the 747 clones examined represent approximately 1/1000 of the bat genome. Two clones (Rp59 and Rp435) were identified as hypervariable, in that they were present in the ingroup (*R. pumilio*) but absent in the outgroup (*A. jamaicensis*). The low intensity of hybridization of these two clones suggests that they are members of a family of repeats characterized by low copy number. Four additional clones (Rp418, Rp607, Rp612, and Rp627) showed more intense hybridization in *R. pumilio* than in *A. jamaicensis* but were not

considered as representing hypervariable clones as these sequences would not be expected to contribute to the hypotheses as outlined in Wichman et al. (1990).

The potential interrelationship (sequence similarity) of these two clones was examined using cross-hybridization experiments. For each clone, bands (DNA fragment) generated by the triple digest of *Bam*HI, *Eco*RI, and *Hin*dIII were used as probes (Fig. 1). In each case, the labeled fragment hybridized to itself and other fragments from that clone; however, no fragment cross-hybridized to any other clone.

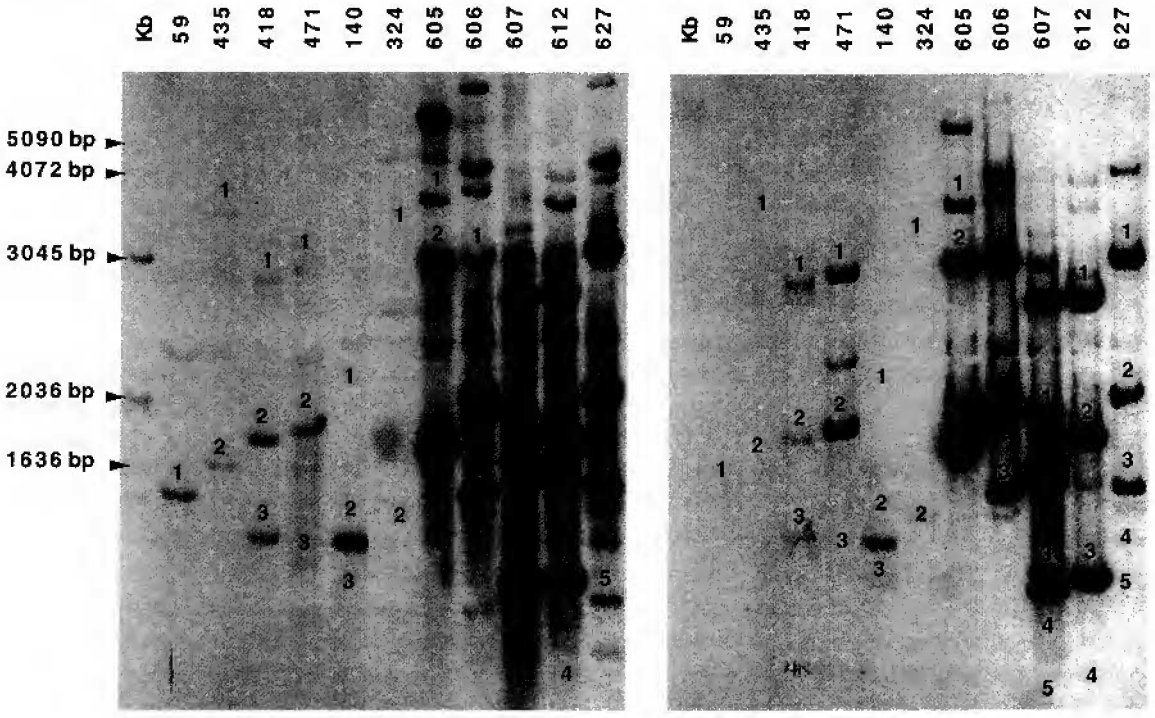

Figure 1. Autoradiogram depicting the phylogenetic screening process for representative clones (DNA sequences) isolated from a library constructed from genomic DNA obtained from *Rhinophylla pumilio*. Clones were triple digested with *Bam*HI, *Eco*RI, and *Hin*dIII restriction enzymes and were then hybridized to a radioactively-labeled probe constructed from total genomic DNA from *Rhinophylla pumilio* (left) and *Artibeus jamaicensis* (right). Only hybridization to repetitive elements is visible under these conditions.

DISCUSSION

Seven hundred forty seven inserts were examined in this study. Of these, 103 did not show detectable hybridization to total genomic DNA and thus are thought to contain single or low copy sequences; consequently, they were removed from further consideration. The majority of the remaining clones (638 of 644) contained repetitive sequences that do not appear to show different patterns of evolution since the divergence of *R. pumilio* and *A. jamaicensis*. Of the remaining six clones, only two meet the criteria of hypervariable as defined by Wichman et al. (1991). These two DNA sequences appear to have originated since *R. pumilio* and *A. jamaicensis* shared a common ancestor. In addition, these clones appear to represent different DNA sequence families, as they share no apparent sequence similarity in cross-hybridization experiments. Alternatively, these sequences could contain portions of a larger repeat.

Results of this study (Table 1) were similar to that obtained from the phylogenetic screening of the *Macrotus* genome (Bradley and Wichman, 1994). Both genomes contained a paucity of hypervariable clones and families of hypervariable sequences. However, these data differed substantially from similar studies of rodents (Wichman et al., 1985; 1990), primates (Lloyd et al., 1987), and equids (Wichman et al., 1990; 1991).

Table 1. Comparison of results from phylogenetic screening efforts of rodents (Wichman et al., 1985; 1990), equids (Wichman et al., 1990; 1991), primates (Lloyd et al., 1987), and bats (Bradley and Wichman, 1994; this study).

Taxon	% Genome Examined	# Variable Clones	# Variable Families	Average Genome Size of Order (Mb)
Peromyscus	0.1	11	4	3400
Equus	0.1	34	6	3019
Homo	0.17	20	3	3000
Macrotus	0.1	1	1	1890
Rhinophylla	0.1	2	2	1890

Interpretation of the data generated from this study require the rejection of the prediction by Wichman et al. (1991) that lineages that undergo rapid karyotypic change would have multiple families of tandemly repeated sequences. However in rejecting this hypothesis, three alternative scenarios must be examined. First, it may be that bats possess fewer repetitive sequences than do other mammals. This possibility is supported by the observation of Manfredi Romanini (1985) and Burton et al. (1989) that bats possess approximately 60-80% of the DNA found in typical mammalian species. Although, the hypothesis that genome size is proportional to the number of repeats or repeat activity has been debated recently (Mouse Genome Sequencing Consortium, 2002).

Second, our phylogenetic screening procedure may not have detected the presence of some hypervariable sequences. In this study, we choose *A. jamaicensis* as the outgroup taxon for evaluating the evolution of genomic sequences in the rapidly evolving genome of *R. pumilio*. It may be that the genome of *A. jamaicensis* contains many of the potentially rapidly evolving sequences found in the *R. pumilio* genome. It is known that *A. jamaicensis* possesses a moderately evolving genome as calculated from the accumulation of chromosomal rearrangements since its divergence from the base of the Phyllostomid clade (Baker et al., 1989). Additionally, it may be that the ingroup taxon is too closely related to the outgroup taxon. What may be more valuable is a comparison of *Rhinophylla* to *Macrotus*. This would provide for a more conservative test.

Third, for bats, it may be that tandemly repeated sequences do not drive chromosomal evolution as is hypothesized for equids (Wichman et al., 1990, 1991). Alternatively, some other molecular mechanism is responsible for this pattern of karyotypic megaevolution. For example, the rate of insertions/deletions or substitutions is very high in repeats of chiropterans and thus these divergent families of repeats would be missed by our stringent hybridization conditions. The comparison of the mouse and human genome showed that substitution rates among repeats vary significantly. Of course, the longer the repeat the less the effect of the substitution rate on the hybridization. Sequencing the hypervariable clones would give us an indication of the type and length of the repeat (Mouse Genome Sequencing Consortium, 2002). Another possibility is that sequences with a large number of repeats are difficult to clone. The selection of 4-6kb fragment sizes could have also contributed to the loss of repeat sequences.

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